AEM
Journals.ASM.org

Sharply Tuned pH Response of Genetic Competence Regulation in *Streptococcus mutans*: a Microfluidic Study of the Environmental Sensitivity of *comX*

Minjun Son,[a] Delaram Ghoreishi,[a] Sang-Joon Ahn,[b] Robert A. Burne,[b] Stephen J. Hagen[a]

Department of Physics, University of Florida, Gainesville, Florida, USA[a]; Department of Oral Biology, University of Florida, Gainesville, Florida, USA[b]

Genetic competence in *Streptococcus mutans* is a transient state that is regulated in response to multiple environmental inputs. These include extracellular pH and the concentrations of two secreted peptides, designated CSP (competence-stimulating peptide) and XIP (*comX*-inducing peptide). The role of environmental cues in regulating competence can be difficult to disentangle from the effects of the organism's physiological state and its chemical modification of its environment. We used microfluidics to control the extracellular environment and study the activation of the key competence gene *comX*. We find that the *comX* promoter (P_{comX}) responds to XIP or CSP only when the extracellular pH lies within a narrow window, about 1 pH unit wide, near pH 7. Within this pH range, CSP elicits a strong P_{comX} response from a subpopulation of cells, whereas outside this range the proportion of cells expressing *comX* declines sharply. Likewise, P_{comX} is most sensitive to XIP only within a narrow pH window. While previous work suggested that *comX* may become refractory to CSP or XIP stimulus as cells exit early exponential phase, our microfluidic data show that extracellular pH dominates in determining sensitivity to XIP and CSP. The data are most consistent with an effect of pH on the ComR/ComS system, which has direct control over transcription of *comX* in *S. mutans*.

Genetic competence is a transient physiological state during which a bacterial cell is able to internalize DNA from its environment. Competence occurs in many bacterial species but was first described in the streptococci, where its regulation has been the subject of intensive study (1, 2). In the oral pathogen *Streptococcus mutans*, competence is important not only because it contributes to genetic diversity but also because its regulation is closely intertwined with the manifestation of virulence-related behaviors, including bacteriocin production, biofilm formation, tolerance of low pH, and carbohydrate catabolism (3–7). *S. mutans* regulates competence in part through two secreted quorum-sensing peptides, designated competence-stimulating peptide (CSP) and *comX*-inducing peptide (XIP). Interestingly, the activity of these peptides depends on environmental parameters, including pH, carbohydrate, and media (8–11). Through mechanisms that are not well understood, the competence regulon integrates the peptide signals with environmental and internal parameters (12, 13) to trigger a transient state of competence during early exponential growth phase.

The interaction of the extracellular environment with competence and related virulence behaviors is important in the context of oral biofilms. Heterogeneous local environments of pH and oxygen/redox, carbohydrate, and secreted-peptide concentrations in a biofilm could potentially lead to spatial variations in virulence gene expression in *S. mutans* (14–17). pH is particularly important because the fermentation of carbohydrates by *S. mutans* generates acids that can rapidly modify the pH of the environment. The pH in a biofilm can locally fall below pH 5.0 (15, 16), with large variations in pH reported to occur over distance scales as small as a few micrometers (18). As the ability to both generate and tolerate acidic conditions is itself an essential characteristic of the virulence of *S. mutans* (19, 20), it is critical to understand how extracellular pH interacts with the regulation of competence and virulence.

At the center of competence regulation in *S. mutans* is *comX* (also known as *sigX*), which encodes an alternative sigma factor required for the expression of late competence genes. ComX is absolutely required for transformation (21, 22). The transcription of *comX* is activated by the ComR/ComS system (23): *comS* encodes the precursor for the 7-residue peptide XIP, which interacts with ComR to form a complex that is the transcriptional activator of both *comX* and *comS*. In chemically defined media, expression of *comX* can be directly activated by exogenous XIP, which is internalized and forms a complex with the ComR protein that binds to the *comX* promoter (P_{comX}) region.

In planktonic and biofilm cultures supplied with complex media, *comX* expression and transformability can be induced by the addition of exogenous CSP. CSP is a 21-residue peptide derived from posttranslational processing and secretion by ComAB. Further extracellular processing of CSP by the SepM protease yields an 18-amino-acid peptide that is believed to be the most active form of CSP (24). The mechanism by which extracellular CSP causes upregulation of *comX* is indirect and more complex than is the case for XIP. Extracellular CSP is detected by the two-component signal transduction system ComD/ComE, leading to phos-

Received 29 April 2015 **Accepted** 29 May 2015

Accepted manuscript posted online 12 June 2015

Citation Son M, Ghoreishi D, Ahn S-J, Burne RA, Hagen SJ. 2015. Sharply tuned pH response of genetic competence regulation in *Streptococcus mutans*: a microfluidic study of the environmental sensitivity of *comX*. Appl Environ Microbiol 81:5622–5631. doi:10.1128/AEM.01421-15.

Editor: M. Kivisaar

Address correspondence to Stephen J. Hagen, sjhagen@ufl.edu.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01421-15.

doi:10.1128/AEM.01421-15

phorylation of ComE (to ComE-P), which functions as an activator of genes encoding a variety of bacteriocins. ComE-P also stimulates the ComR/ComS system through an unknown mechanism. The ComR/ComS system provides positive autofeedback regulation of *comS*, and this feedback is evidently stimulated by CSP. As a result, CSP triggers bimodal activation of the ComR/ComS system as well as *comX*. That is, under stimulation by CSP, a subpopulation of *S. mutans* cells (typically 1 to 10%) activates *comX* while the rest of the population does not (10, 25).

Finally, CSP is effective in stimulating P_{comX} only in complex (peptide-rich) growth media, whereas XIP is effective only in chemically defined media such as FMC (10, 23, 26, 27). We previously posited that the connection between growth medium and the P_{comX} response to XIP or CSP can be understood in terms of direct versus autofeedback stimulation of the ComR/ComS system (10).

Guo et al. recently reported that pH plays an important role in the regulation of competence in *S. mutans* (11). In a study using batch-grown, planktonic cultures, natural competence development and competence induction by XIP or CSP were optimal during early exponential phase (optical density [OD] = 0.2). However, cells later in the growth cycle (e.g., OD = 0.4 or 0.8) were unresponsive to XIP or CSP, as assessed by reporter gene fusions and transformation efficiency assays (11). Because *S. mutans* acidifies its environment during growth, Guo et al. tested whether restoring mid- or late-exponential-phase cultures (OD = 0.4 or 0.8, respectively) to neutral pH or exchanging the supernatants for fresh media could restore *com* gene expression and transformation. Neutralizing the pH largely restored the response to XIP in cells grown to late exponential (but not mid-exponential) phase. However, cells in mid- and late exponential phase showed a reduction in *com* gene response to CSP that could not be restored by neutralizing the pH. These data clearly showed an important role for pH in *S. mutans* competence regulation. However, they could also indicate that internal cellular mechanisms activated during mid-exponential phase or inhibitory substances accumulating in the medium also limit the duration of the competent state. *S. mutans* modifies its environment during growth through production of acid, depletion or generation of signal peptides and other factors, formation of biofilms, and other behaviors. It is important therefore to establish more clearly whether changes in the cells and their environment play an important role in inhibiting the activation of *comX* later in growth.

In studying planktonic or biofilm cultures, it is difficult to unravel the influence that environmental factors such as pH, signal peptides, growth phase, and medium composition have on gene regulation. In the present study, we distinguish the effect of pH from other environmental effects by studying cells that are adhered in microfluidic growth chambers, which supply a continuous and stable flow of media that can be precisely defined and modulated. The activation of *S. mutans* competence genes in these controlled environments can be studied at the single-cell level using fluorescent protein reporters. Our laboratory groups recently showed that microfluidic methods can clarify the role of signals, signal transduction systems, and other regulatory circuits in the regulation of *S. mutans* competence. Here fine microfluidic control of the environment allows us to determine how the extracellular pH modulates the responsiveness of the competence network to stimulation by CSP and XIP.

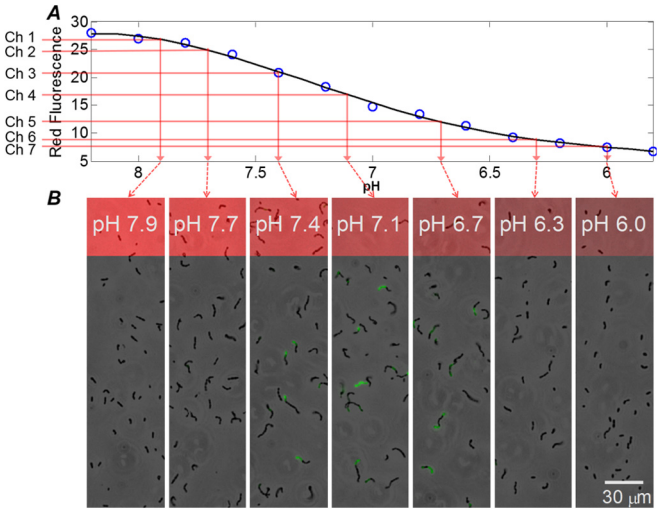

FIG 1 Individual cells of *S. mutans*, which grows in short chains, were observed within a microfluidic device containing seven parallel flow chambers that supply media at different, stable pHs. The flow in each chamber was generated by mixing inlet media of different pHs, and pH was verified by measuring the red fluorescence of a pH-sensitive tracer dye. (A) The dye fluorescence versus pH was measured in independent measurements on bulk samples, generating the calibration points shown in blue. The curve is a polynomial fit to the calibration points. The arrows relate the relative fluorescence of the dye in the microfluidic chambers to locations on the calibration curve. (B) Images of microfluidic flow chambers containing adhered *S. mutans* at different pHs. P_{comX} activity was detected using a GFP reporter. All channels were provided with 1 μM synthetic CSP in complex growth medium (see Materials and Methods). The images are a partial overlay of the tracer dye fluorescence in the chambers (red), the GFP fluorescence of the cells adhered in the flow chambers (green), and phase-contrast (gray).

MATERIALS AND METHODS

Construction of reporter strains. The construction of the P_{comX}-*gfp* reporter strain was described previously (10). The P_{cipB}-*gfp* reporter strain was constructed by replacing P_{comX} by P_{cipB} (SMU.1914c). Briefly, a 214-bp region comprising P_{cipB} was PCR amplified with primers TCATG GATTAAGCTTAAAAAGTAAT and TGTATTCATACTAGTAATACCC CTT, which incorporated HindIII and SpeI sites, respectively, and was cloned in front of the superfolder green fluorescent protein (sGFP) gene in pDL278. The resulting construct was transformed into wild-type strain UA159. For constitutive expression of *gfp*, the region containing a *sarA* P1 promoter (a constitutive promoter of multiple virulence determinants in *Staphylococcus aureus*) and the sGFP gene was PCR amplified from pCM11 and cloned into pDL278 (28, 29).

Microfluidic experiments. Microfluidic fabrication and related procedures were described previously (10). The microfluidic device has three inlet channels for media and seven parallel flow chambers in which *S. mutans* cells adhere (see Fig. S1 in the supplemental material). To create the pH gradient across the seven chambers, we inject at the inlet channels media at three pH values, where the media contain 2 to 3 μM pH-sensitive red fluorophore (SNARF-5F carboxylic acid; Molecular Probes). The device mixes the inlet flows to deliver media at different pH values into the cell chambers. We verified the pH of the media within each chamber by measuring the red SNARF fluorescence of each chamber and comparing it to a calibration curve (Fig. 1).

The SNARF calibration curve was obtained by dissolving 2 to 3 μM dye in fresh medium at different pH values and loading this mixture into a commercial channel slide (Ibidi; 80661). At each of the pH values, three red fluorescence images were collected using the same Nikon Cy3 HYQ filter as used for live cell studies. The mean of three fluorescence values was plotted and fit to a polynomial over the range of pH 8.2 to 5.8 (Fig. 1).

P_{comX}-*gfp* or P_{cipB}-*gfp* strains of *S. mutans* were grown to the desired OD (OD = 0.1, 0.4, or 0.8 at 600 nm), ultrasonicated for 10 to 30 s (Fisher FB120 ultrasonicator at 30% amplitude), and then loaded into the chambers of the flow device, which was installed on the stage of an inverted microscope in an anaerobic chamber at 37°C. The fluid flow in the flow chambers (see Fig. S1 in the supplemental material) was then switched on to provide the stable pH gradient. The cells were imaged in phase-contrast, GFP fluorescence, and red (SNARF) fluorescence every hour for 2 to 3 h. The fluorescence of the *gfp*-active cells grows monotonically over this period as GFP accumulates. Imaging was computer controlled using a motorized stage, shutter, and cooled charge-coupled device (CCD) camera under the control of custom software. To verify that our GFP signal was reliable over the full pH range of interest, we also loaded a strain containing a constitutive *gfp* reporter into the chambers and verified that the GFP fluorescence is virtually unchanged over the pH range of interest (see Fig. S2).

Fresh media were used in some microfluidic experiments, while filtered supernatants were used in others. For all CSP studies, the fresh medium was 1/3 brain heart infusion (BHI) medium and 2/3 FMC medium (vol/vol), while for all XIP studies the fresh medium was FMC (30). It was necessary to dilute the BHI in the CSP studies because pure BHI medium produces a strong green fluorescence background signal. For microfluidic experiments with supernatants, cell cultures that were inoculated in the appropriate fresh media were centrifuged to separate the supernatant from the cell pellet and filtered (0.22 μm) and 2 to 3 μM SNARF dye was added. For pH gradient studies, the pH of the media or supernatants to be supplied was adjusted to 6, 7, or 8 by addition of a small amount of HCl or NaOH and the appropriate signal peptide was then added at the concentration of 1 μM synthetic CSP or 500 nM synthetic XIP. The three pH-adjusted media were then combined within the microfluidic device to produce the pH gradient, as shown in Fig. 1. For XIP or CSP concentration gradient experiments, the media entering the microfluidic device were corrected to pH 6 or 6.3 but the three inlet streams contained XIP (0, 5, 10 μM) or CSP (0, 3, 6 μM) concentrations that mixed to generate a linear gradient. To permit monitoring of XIP or CSP concentrations in peptide gradient experiments, a red fluorescent tracer (sulforhodamine 101; 50 to 100 ng ml^{-1}) was also added to each input medium in proportion to its XIP or CSP concentration (10).

Bulk culture experiments. We performed two types of experiments on P_{comX} activity in pH-adjusted bulk cultures of *S. mutans*: continuous pH correction and single-time-point pH correction. In both types, *S. mutans* cultures were grown to an OD of 0.1 or 0.4 and then divided into 4 samples. The pH of the samples was adjusted to 7.9, 7, 6.4, or 6 by adding small amounts of 2 N NaOH or HCl. XIP at 500 nM or CSP at 1 μM was then added.

In the continuous adjustment experiment, the pH of each sample was subsequently maintained within ±0.1 pH unit of its initial value through continuous pH adjustments while the sample was kept in a water bath at 37°C. The sample pH was measured every 10 to 15 min, and if the pH had fallen 0.1 unit below its initial value small aliquots (1 to 2 μl) of 2 N NaOH were added until the pH was 0.1 unit above the initial value. As cultures entered mid-exponential phase, more 2 N NaOH was needed in order to provide the adjustment. In the single-time-point adjustment experiment, the samples were incubated at 37°C immediately after addition of 500 nM XIP or 1 μM CSP, without further pH adjustment.

For both cases, cells were extracted 2 h after addition of the peptide, briefly ultrasonicated as above, dispersed onto a slide, and then imaged in phase-contrast and GFP fluorescence. Imaging employed the same illumination and exposure settings that were used for the microfluidic studies.

Single-cell image analysis. The expression of P_{comX}-*gfp* or P_{cipB}-*gfp* in individual cells was quantified by analyzing phase-contrast and GFP fluorescence images of individual cells with custom-developed Matlab software. The analysis method has been described previously (31). Briefly, the method correlates the intensity of the phase-contrast image of an individual cell with the brightness of its GFP fluorescence image, giving a unitless parameter (denoted *R*) that is proportional to the concentration of GFP within the cell. *R* is therefore a measure of the *gfp* expression activity in that cell. The histograms shown in the data figures indicate the distribution of *R* values across a sampling of cells within the flow chamber.

RESULTS

XIP and CSP activate P_{comX} in a narrow pH range. The activation of P_{comX} by XIP or CSP was measured as a function of extracellular pH in *S. mutans* cells adhered within microfluidic flow chambers as shown in Fig. 1. *S. mutans* grown to early (OD = 0.1 to 0.2 at 600 nm), mid (OD = 0.4)-, or late (OD = 0.8) exponential phase (see Materials and Methods) were loaded into the chambers and supplied with a continuous flow of either fresh media or supernatant fluids taken from planktonic cultures at different growth phases. Synthetic CSP or XIP was added to the media, which were adjusted to a different pH in each flow chamber (Fig. 1). The flow rate for the media was sufficient to refresh the fluid in each chamber approximately every 10 s, such that endogenously produced XIP, CSP, or other secretions should be rapidly cleared from the chambers. Because small peptides block XIP signaling but are required for CSP signaling, all experiments with XIP were conducted using the chemically defined, peptide-free medium FMC and all experiments with CSP were conducted with a mixture of 1/3 complex (BHI) medium and 2/3 FMC. Activation of P_{comX} was monitored through expression of a P_{comX}-*gfp* reporter, and pH was verified by measuring the red fluorescence of a tracer dye (see Materials and Methods). Cells were typically exposed to flow for 2 h prior to collection of phase-contrast and GFP fluorescence images. During this period, most cells remained adhered within the chambers and some cell division occurred. Population-wide patterns of *gfp* activation in the adhered cells did not change during observation, although GFP accumulated monotonically in the *gfp*-active cells. Therefore, an optimum trade-off between bright fluorescence and crowding of the flow chamber occurred at 2 h. Images of several hundred cells within each chamber were then collected and analyzed to generate histograms of GFP expression levels in the population, as described in Materials and Methods.

To begin to explore the effects of pH on XIP and CSP signaling, a P_{comX}-*gfp* strain that was grown to early exponential phase (OD = 0.1) was introduced into the microfluidic device and provided with a flow of fresh medium containing either 1 μM CSP or 500 nM XIP. All chambers received the same concentration of signal peptide, while the media in different chambers spanned pH 6 to pH 8 (Fig. 2). As expected (10), CSP elicited a bimodal *comX* response at neutral pH values: a portion of the population exhibited only baseline P_{comX} activity, whereas P_{comX} was upregulated roughly 100-fold in a subpopulation of cells. However, this response occurred within a very narrow pH range, with optimal induction near pH 7.1. At higher or lower pH values, the fraction of cells activating *comX* decreased sharply, even though the level of expression in the activated cells remained high. Also as expected (10), XIP elicited a unimodal response from P_{comX} at neutral pH values. At pH 7.1, virtually all cells in the population activated P_{comX} in the presence of XIP. However, P_{comX} activity was reduced in chambers that were more acidic or basic, with little more than baseline activity detectable at pH 6.0.

Figure 2B shows the mean P_{comX} activity versus pH. Although the bimodal response to CSP resulted in overall lower mean P_{comX} activity than observed for XIP, the pH dependence of the mean

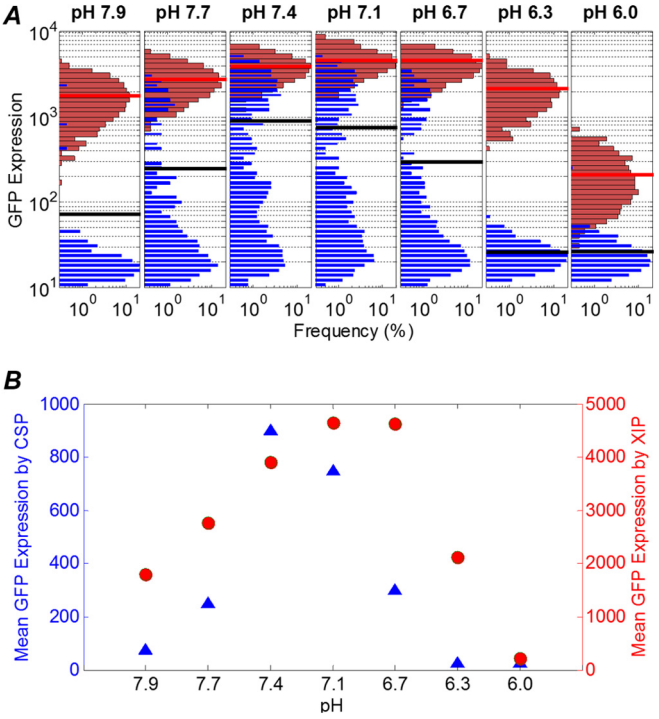

FIG 2 P_{comX} activity in cells grown to early exponential phase (OD = 0.1) and provided 1 μM CSP or 500 nM XIP in flowing fresh media at the indicated pH values. (A) Histogram showing GFP concentration measured in individual cells carrying a P_{comX}-*gfp* reporter 2 h after introduction of flow of media containing CSP (blue) or XIP (red). Approximately 700 to 1,000 cells were imaged and analyzed at each pH condition to obtain the GFP concentration within each cell (see Materials and Methods). The horizontal axis shows the percentage of cells in each flow channel for which the GFP was expressed at the indicated level. (The baseline corresponding to "dark" cells is approximately 10 to 30 units.) Bimodal activation of P_{comX} by CSP appears as a double-peaked shape of the blue histograms near pH 7. Population mean GFP expression for stimulation by CSP (black bars) and XIP (bright red bars) is also shown. (B) Mean GFP concentration following activation of the P_{comX}-*gfp* reporter by CSP (blue) or XIP (red), as evaluated from the single-cell histograms in panel A. The mean CSP and XIP activation is shown on different vertical scales, as CSP induces P_{comX} activity in only a subpopulation of cells.

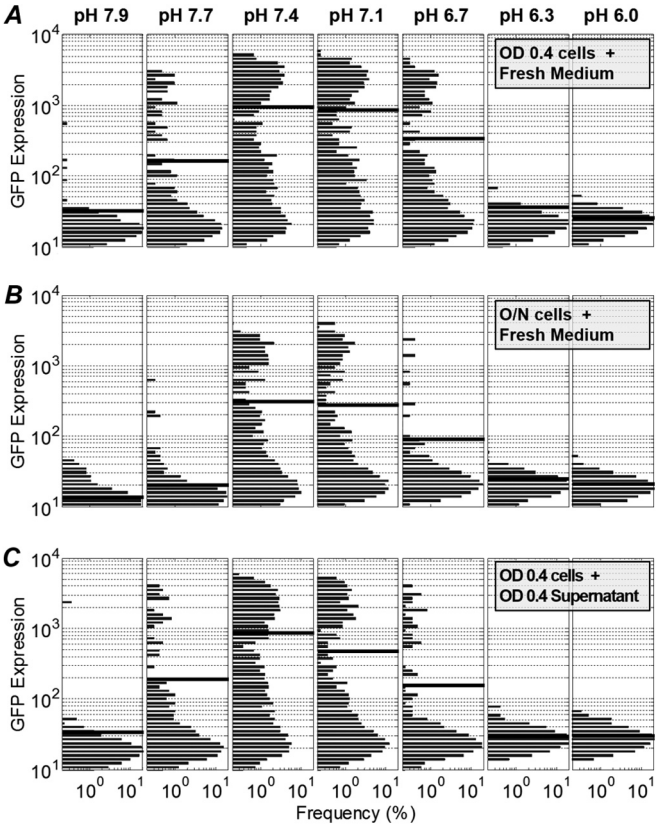

FIG 3 Effect of growth phase and media on the pH dependence of the activation of P_{comX} by CSP in the microfluidic chambers. Cells at various growth phases were supplied with either fresh media or their own filter-sterilized, pH-adjusted, glucose-supplemented supernatant fluids containing 1 μM exogenous CSP. A black bar indicates the population mean of each histogram. (A) Cells from mid-exponential phase (OD = 0.4) were supplied with fresh medium. (B) Cells from an overnight culture (OD ≥ 1.2) were supplied with fresh medium. (C) Cells from mid-exponential phase (OD = 0.4) were supplied with their own pH-corrected supernatant fluids. Figure S5 in the supplemental material shows similar results for late-exponential cells supplied with their own supernatants.

P_{comX} activity showed generally similar, peaked behaviors near pH 7.1 for XIP and CSP. Peak sensitivity to XIP occurred over a slightly broader pH range, more than 1 pH unit wide, while peak sensitivity to CSP occurred at a slightly higher pH and in a narrower window than for XIP.

pH influences CSP sensitivity in mid- and late-exponential-phase cells and supernatants. A study of planktonic *S. mutans* growing in batch cultures found a reduced *comX* response to XIP and, to a lesser extent, CSP during mid- and late exponential phase, somewhat independent of extracellular pH (11). To test if cells in microfluidic chambers exhibited similar behaviors, *S. mutans* was grown to mid-exponential (OD = 0.4) or to late stationary phase (overnight cultures, OD ≥ 1.2). The cells were loaded into flow chambers and supplied with CSP in fresh medium that was adjusted to different pH values. The pattern of P_{comX} expression (Fig. 3A and B) was similar to that for early exponential cells (Fig. 2); cells grown to mid-exponential phase or overnight responded to CSP within a narrow pH interval near 7.1. This indicates either that the physiologic state of the cells does not dictate the pH dependence of the response to CSP or else that cells loaded into the microfluidic chamber adapt rapidly enough to the new environment that they respond differently than cells cultured batch-wise in bulk. Adaptation could occur through rapid clearance of an inhibitory substance present in the batch culture or from a purely internal (genetic or physiologic) response to the flow chamber environment.

We tested whether batch cultures contained a substance that inhibits CSP responsiveness in cells that have exited early exponential phase. For this experiment, we collected supernatants from mid (OD = 0.4)- and late (OD = 0.8)-exponential-phase cultures, clarified them by centrifugation, and filter sterilized the supernatant fluid. To compensate for the depletion of the carbohydrate source, 10 mM glucose was added. CSP was then added to a final concentration of 1 μM to the sterile supernatants, and the pH was adjusted as above. These mixtures were then supplied via the flow chambers as input media to the same mid- or late-exponential-phase cells from which the supernatants were obtained. Figure 3C shows that in chambers where the mid-exponential-phase cells were supplied with their own supernatants, the full *comX* response was restored by neutralization of pH and the pH

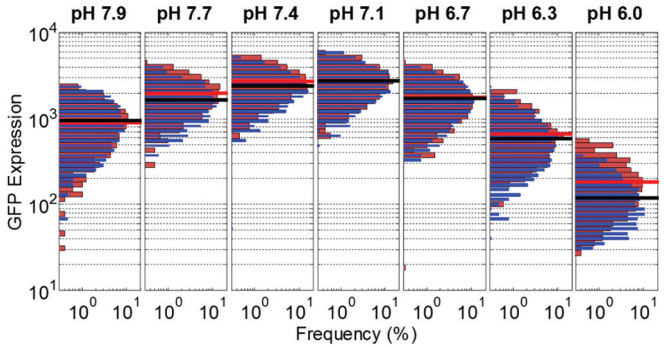

FIG 4 Effect of culture supernatants on the pH dependence of P_{comX} activation by XIP. Cells were grown to an OD of 0.2 (red) or 0.8 (blue) and then provided 500 nM XIP in a flow of their own filtered and pH-adjusted supernatant. P_{comX} activity was measured after 2 h. The histograms indicate GFP expression levels measured in individual cells. Red and black bars indicate mean expression levels of cells that were grown to ODs of 0.2 (red) and 0.8 (black). GFP expression levels (mean ± standard deviation) under the two experimental conditions at pH 7.4 are 2,400 ± 900 (OD = 0.8) and 2,700 ± 1,000 (OD = 0.2); levels at pH 7.1 are 2,800 ± 1,100 (OD = 0.8) and 2,800 ± 1,100 (OD = 0.2); levels at pH 6.7 are 1,700 ± 800 (OD = 0.8) and 1,800 ± 900 (OD = 0.2); and levels at pH 6.0 are 120 ± 70 (OD = 0.8) and 200 ± 100 (OD = 0.2).

dependence was nearly equivalent to that seen in fresh media. When late-exponential-phase cells were supplied with their own supernatants, the *comX* response was also restored at neutral pH (see Fig. S5 in the supplemental material); however, the fraction of cells activating P_{comX} was smaller than the fraction of early- or mid-exponential-phase cells, and the level of *gfp* expression in the activated cells was very slightly lower. Overall, however, the pH dependence of the P_{comX} response to CSP was retained in all populations and combinations of cells and supernatants tested. These data imply that mid- and late-exponential-phase culture supernatants do not contain substances that inhibit *comX* activation. Restoring the pH to neutral value is sufficient to restore the *comX* response.

pH controls XIP sensitivity in mid- and late-exponential-phase cells. We also tested whether the growth phase of the cells or supernatants from cells in different growth phases affected the activation of P_{comX} by XIP. *S. mutans* cells grown in batch cultures to an OD of 0.2 or 0.8 were loaded into the microfluidic chambers and supplied with a flow of their supernatants, which had been filter sterilized, pH adjusted, and supplemented with 500 nM XIP. Additionally, 10 mM glucose was added to the supernatant of the OD 0.8 culture. In both cases, the induction of P_{comX} by XIP was completely restored at pH 7.1 (Fig. 4), and the same overall profile in response to pH that was observed in early-exponential-phase cells supplied with fresh media was observed under these conditions (Fig. 2). Thus, in contrast to results observed in batch-grown cultures, the effect of pH on XIP induction of *comX* did not depend on the growth phase of the cells in the chambers or the cultures from which the supernatants were obtained.

P_{comX} response to XIP and CSP in pH-adjusted batch cultures. Adhesion of bacteria to surfaces, even inert surfaces such as glass or plastic, has been shown to trigger changes in gene expression (32). To verify that our findings were not attributable to adherence of *S. mutans* to the walls of the microfluidic chambers or to the microfluidic environment otherwise, we repeated a subset of the pH experiments using batch-grown cells drawn from bulk liquid cultures. In these cases, XIP or CSP was provided to planktonic *S. mutans* in bulk cultures in which the pH had been adjusted to the desired levels. After 2 h of incubation with XIP or CSP, cells were drawn from the culture and dispersed on a glass slide, and their P_{comX}-*gfp* activity was immediately analyzed by fluorescence microscopy as in the microfluidic experiments.

We used two different methods of pH adjustment in these bulk cultures. In the first method, the pH of a liquid culture was monitored and adjusted every 15 min by addition of NaOH (see Materials and Methods). This maintained a nearly constant pH (±0.1 unit) while the cells responded to exogenous CSP or XIP. In the second method, the pH of the liquid cultures was adjusted only once, prior to adding CSP or XIP. The pH was then allowed to drift downward as the culture grew and responded to the CSP or XIP. For the continuous-adjustment study, four liquid cultures were adjusted to pH 7.9, 7.0, 6.4, or 6.0. The signal peptide (1 μM CSP or 500 nM XIP) was then added, and the pH of these samples was monitored and maintained at the initial values. GFP fluorescence was measured after 2 h. The results were similar to those observed in the microfluidic experiments. Figure 5A shows that, when the pH of an OD 0.1 culture was adjusted continuously, both XIP and CSP elicited a strong P_{comX} response at pH 7.0. Similarly, for cultures that were initially provided with CSP or XIP at an OD of 0.4, the strongest response was observed in the samples that had been maintained at pH 7.0 (Fig. 5B).

As in the microfluidic experiments, CSP elicited little or no P_{comX} response when the pH was maintained at 7.9, 6.4, or 6.0. Interestingly, XIP produced robust P_{comX} activity in the pH 6 sample. However, that P_{comX} response was still 4-fold weaker than the response at pH 7.0. Figure 5B shows that, when an OD 0.4 culture was continuously maintained at pH 7.0 in the presence of CSP, a smaller proportion of cells activated P_{comX} than in the microfluidic studies, but P_{comX} responded only in the pH 7.0 sample. The fact that a smaller proportion of cells in this culture responded to CSP, relative to the OD 0.1 culture, may reflect lower nutrient availability. It could potentially also result from some depletion of small peptides in the complex media; we have previously argued that these peptides drive the ComR/ComS feedback mechanism (10).

In order to evaluate the effect on P_{comX} expression of allowing the cells to modify the pH of their environment through growth and glycolysis, we performed studies in which the pH was adjusted only once, prior to the 2 h of incubation. Here the pH of four liquid cultures was initially adjusted to 7.9, 7.0, 6.4, or 6.0 immediately prior to addition of XIP or CSP. Subsequently, the pH of these cultures drifted rapidly downward (see Fig. S3B in the supplemental material), so the pH at the time of GFP measurement was lower than at the time when XIP or CSP was added. The pH at measurement was also lower than in the experiments in which pH was continuously adjusted during the incubation.

As expected, the samples that were initially adjusted to more-alkaline pH values showed a robust P_{comX} response after 2 h. For cells grown to an OD of 0.4 before the single pH adjustment (Fig. 5C), only the sample adjusted to pH 7.9 showed appreciable P_{comX} activity in response to CSP. Similarly, for an OD 0.4 culture that was provided with exogenous XIP, the sample that had been initially adjusted to pH 7.9 showed the strongest response to XIP (Fig. 5C).

Finally, we tested the kinetics of the pH suppression of CSP and XIP sensitivity in batch cultures. In these studies (see Fig. S4 in the supplemental material), the pH of a batch culture in early expo-

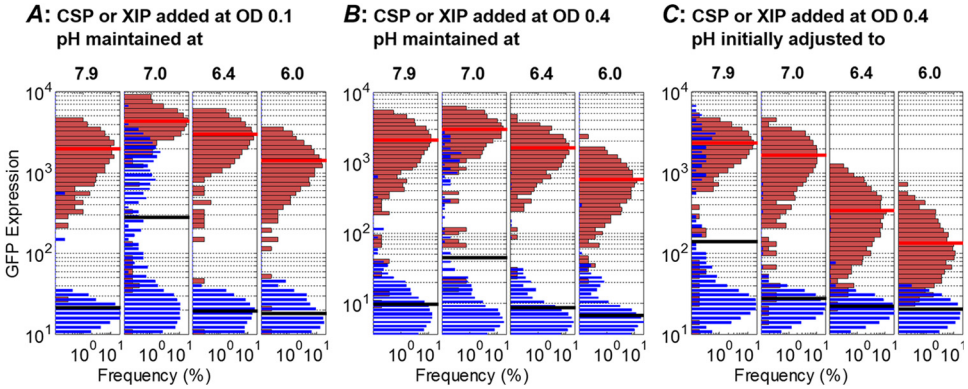

FIG 5 Effect of pH stabilization and pH drift on P_{comX} activity of bulk cultures of *S. mutans*. Culture pH was either adjusted to the indicated values continuously during incubation with CSP or XIP (A and B) or else was adjusted only once prior to the addition of CSP or XIP (C) (see Materials and Methods). Histograms indicate P_{comX} reporter activity measured in individual cells by GFP fluorescence after 2 h of incubation. Bright red and black bars indicate population means of the histograms. (A) The pH of a culture initially at an OD of 0.1 was continuously monitored and adjusted to the indicated value for 2 h following addition of 1 μM CSP (blue) or 500 nM XIP (red). (B) The pH of an OD 0.4 culture was continuously adjusted following addition of 1 μM CSP (blue) or 500 nM XIP (red). (C) The pH of an OD 0.4 culture was adjusted only once prior to addition of 1 μM CSP (blue) or 500 nM XIP (red).

nential phase was adjusted (once) to pH 5.5 at time (t) zero, whereas CSP or XIP was supplied a different time ($t = \tau$). These data show that the drop in pH inhibited the XIP sensitivity of P_{comX} much more rapidly than it inhibited CSP sensitivity (see Fig. S4). Even if CSP was supplied to an OD 0.1 to 0.2 culture 30 min before the pH was lowered to 5.5, the P_{comX} response after 2 h was reduced. By contrast, when XIP was supplied 30 min before the culture was acidified, a significant P_{comX} response still occurred.

Overcoming P_{comX} inhibition at low pH. To determine whether P_{comX} activity could be restored at low pH, we supplied cells with higher concentrations of CSP or XIP at low pH. *S. mutans* cells cultured to an OD of 0.1 were loaded into the microfluidic chambers, and all seven chambers were supplied with fresh FMC medium that was adjusted to pH 6.0. Unlike in previous experiments, however, the pH was the same in all seven chambers but the chambers provided different concentrations of XIP, ranging from 0 to 10 μM. Figure 6A shows that, at XIP concentrations greater than approximately 3 μM, P_{comX} expression was restored nearly to levels observed at pH 7. Comparison of the expression at saturating XIP in Fig. 6 to the expression at neutral pH in Fig. 2 shows that high XIP concentration at pH 6 can restore about 50% of the expression that is observed at neutral pH.

A similar experiment was performed using CSP ranging from 0 to 6 μM in complex medium (1/3 BHI medium and 2/3 FMC) that was adjusted to pH 6.3. Unlike results for XIP, even the highest concentrations of CSP failed to induce P_{comX} activity above baseline (Fig. 6B).

CSP induces unimodal expression of *cipB*, a direct target of ComE, across a wide pH range. As noted above, XIP induces *comX* through the binding of the ComR-XIP complex to the *comX* promoter region, whereas CSP induces *comX* indirectly through the ComDE pathway. The finding that high concentrations of XIP, but not CSP, restored P_{comX} activity at low pH raises the question of whether low pH reduces the ability of the cells to sense CSP via the ComDE pathway. To determine whether the CSP-ComDE signaling pathway remains active at low pH, we tested the effect of pH on the CSP-induced expression of *cipB*. The *cipB* gene encodes a self-acting bacteriocin (mutacin V) that is produced in response to CSP stimulation and is required for CSP-induced transformation of *S. mutans* (33). It is directly activated through interaction of CSP with the ComD sensor kinase, phosphorylation of ComE by ComD, and binding of ComE-P to the *cipB* promoter region (34).

Figure S6 in the supplemental material shows the results of microfluidic studies of *cipB* activation at low pH and in the presence of variable concentrations of CSP. CSP/ComDE signal transduction remained active at low pH. Further, while P_{cipB} showed significant basal activity that exhibited some pH dependence, P_{cipB}

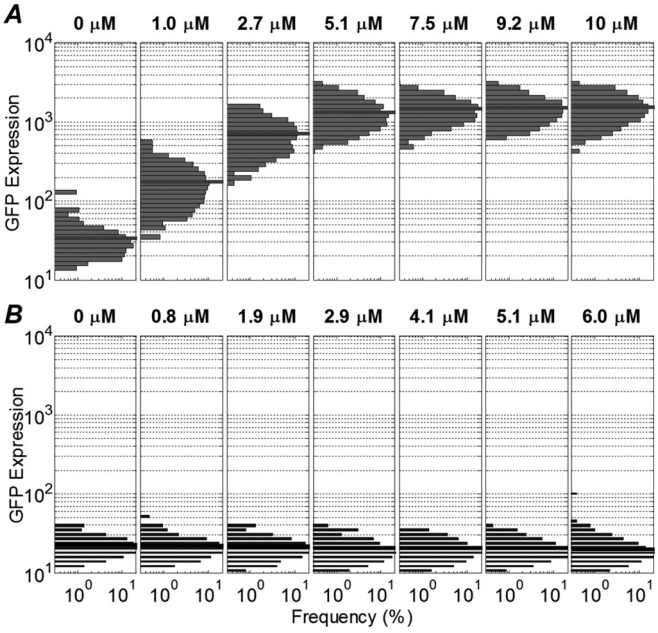

FIG 6 Effect of increased XIP or CSP concentrations on the induction of P_{comX} at low pH. Histograms show population-wide GFP activity in cells grown to an OD of 0.1 and then supplied XIP (0 to 10 μM) or CSP (0 to 6 μM) in a flow of fresh media at low pH. GFP expression was measured after 2 h of flow in the microfluidic chambers. Black bars indicate histogram means. (A) Response of P_{comX} to high concentrations of exogenous XIP at pH 6.0. (B) Response of P_{comX} to increasing concentrations of exogenous CSP at pH 6.3.

expression was significantly enhanced by CSP over a broad pH range. The largely pH-independent function of the CSP/ComDE system suggests that pH primarily controls P_{comX} regulation further downstream in the regulatory pathway.

Finally, the activation of P_{cipB} in Fig. S6 in the supplemental material is unimodal: all cells in the population expressed the reporter gene in response to CSP. The unimodal response of P_{cipB} is consistent with the proposal that bimodality in CSP's activation of P_{comX} arises further downstream in the signaling pathway, probably within the ComRS system (10).

DISCUSSION

Many aspects of *S. mutans* genetic competence, including the factors controlling entry and exit from the competent state, are still not well understood. One challenge has been the difficulty of separating the effects of environmental cues—in particular pH, redox, and nutrient availability—from the effects of the internal genetic and physiologic state of the cell. Nevertheless, extracellular pH clearly plays an important role in the regulation of streptococcal competence. An early study (35) showed that the initial pH of the growth medium affects the onset of the competent state in *Streptococcus pneumoniae*; while other factors were also important, competence occurred earlier and repeatedly in cultures that started with an alkaline pH.

For *S. mutans*, Li et al. (36) found that transformability in biofilms was maximal at pH 7 to 8 and significantly decreased at pH 6.0. No transformation was observed in planktonic cells at pH 6.0. Recent work by Guo et al. (11) began to provide insight into the relationship between the signaling cascades and declining transformability at low pH. Their study focused on the effect of pH and supernatant fluids on competence development in batch-grown cultures of planktonic *S. mutans*. XIP strongly induced *comX* transcription in a defined medium (FMC) at an OD of 0.1, but cells at higher OD were refractory to XIP treatment. The pH of FMC decreases during *S. mutans* growth, from roughly pH 6.9 at an OD of 0.1 to pH 5.6 at an OD of 1.0; Guo et al. found that XIP stimulation of P_{comX} in OD 0.8 cultures could be greatly enhanced by adjusting the cultures back to neutral pH or suspending the cells in fresh media. However, cells at mid-exponential phase (OD = 0.4) were refractory to XIP treatment even when their supernatant was adjusted to neutral pH. Further, when cells grew in complex medium, the response of *comX* to CSP declined after early exponential phase and was not restored when supernatants were adjusted to neutral pH. Such findings could imply that, in addition to pH, secretions into the supernatant or purely internal cellular mechanisms may inhibit expression of *comX* as cells exit early exponential phase. However, while it is clear that pH can influence competence-related signal transduction, studies of batch cultures are subject to inherent limitations associated with depletion of nutrients, accumulation of end products, and the production of competence-inhibiting or -promoting signals. These effects impede the goal of observing the influence of pH alone.

Our data clarify the role of pH in limiting the activation of P_{comX} by XIP and CSP. They show that optimal P_{comX} response requires the pH to lie within a very narrow window, roughly pH 6.7 to 7.7 (Fig. 2). Moreover, pH is far more significant in determining the P_{comX} activity of *S. mutans* than is the history (growth stage) of the cells or of their corresponding supernatants. XIP elicited robust P_{comX} activity in cultures grown to early, mid-, or late exponential phase (OD = 0.2, 0.4, or 0.8, respectively) and in any supernatants from these cultures as long as the pH was held between pH 6.7 and 7.7. Similarly, CSP stimulated P_{comX} in cells in different growth phases and in cells exposed to supernatants from different growth phases, as long as the pH was maintained in the same narrow range. None of our microfluidic data indicate that the responsiveness of P_{comX} to XIP or CSP is diminished by a component (other than low pH) present in mid-exponential or later growth supernatants.

Consequently, it is interesting that the batch culture studies by Guo et al. did not show a clear connection between pH and the action of CSP. Neutralizing the pH of mid-exponential-phase batch cultures had little effect on the sensitivity of P_{comX} to CSP (11). Although that result may appear to contradict the microfluidic data in Fig. 3, we note an important difference in the methodology. Guo et al. made a single adjustment to the pH of a batch culture and then allowed an extended incubation time, during which the pH of the media decreased. In contrast, our microfluidic experiments ensured a stable pH from the time that CSP was added until the P_{comX} reporter was measured. The Guo et al. study is thus more analogous to the batch culture experiments that we performed using a single pH correction followed by 2 h of incubation with XIP or CSP (Fig. 5C). In those studies, the downward drift of extracellular pH (see Fig. S3 in the supplemental material) suppressed P_{comX} response to XIP and (especially) CSP, even in cultures that were initially adjusted to neutral pH.

Other bacterial species possess sophisticated mechanisms for switching off the competence genes and limiting the duration of the competent state, such as the DprA and Clp mechanisms (1, 2). At least some of these mechanisms have been described in *S. mutans* (37). Thus, it is intriguing that pH plays a dominant role in controlling *S. mutans* competence. It provides a simple mechanism for *S. mutans* to limit the duration of its competent state while secreting XIP or CSP. Cultures initially prepared in fresh media will drift downward in pH (see Fig. S3 in the supplemental material), passing through and then exiting the narrow pH window within which XIP and CSP are capable of stimulating P_{comX}. The induced competence will be transient, regardless of whether the cells possess any other mechanism for terminating P_{comX} activity. Whether other bacterial species also regulate competence with such pH sensitivity will not be known until those systems have been studied with sufficiently fine environmental control.

Our data also show that the acidification of its environment by *S. mutans* during growth is a confounding effect in studies of pH response in bulk samples. This finding highlights the importance of environmental cues in the regulation of competence in bulk cultures and biofilms. For example, the HdrRM and BrsRM systems (38, 39) of *S. mutans* can influence cellular behaviors, including competence, in a cell density-dependent fashion. Organisms growing in static cultures or biofilms may potentially activate such density-dependent signaling systems in combination with pH and other environmental signals. Separating these different controlling parameters requires that bulk and biofilm studies be complemented by microfluidic studies that provide precise, stable environmental inputs.

It is curious that both XIP and CSP elicit a response from *comX* only within similar, narrow pH ranges, as these signals act in different ways. One difference is that the response of *comX* to CSP is bimodal, wherein a subpopulation of cells is activated by CSP within the responsive pH window (Fig. 3). In contrast, *comX* re-

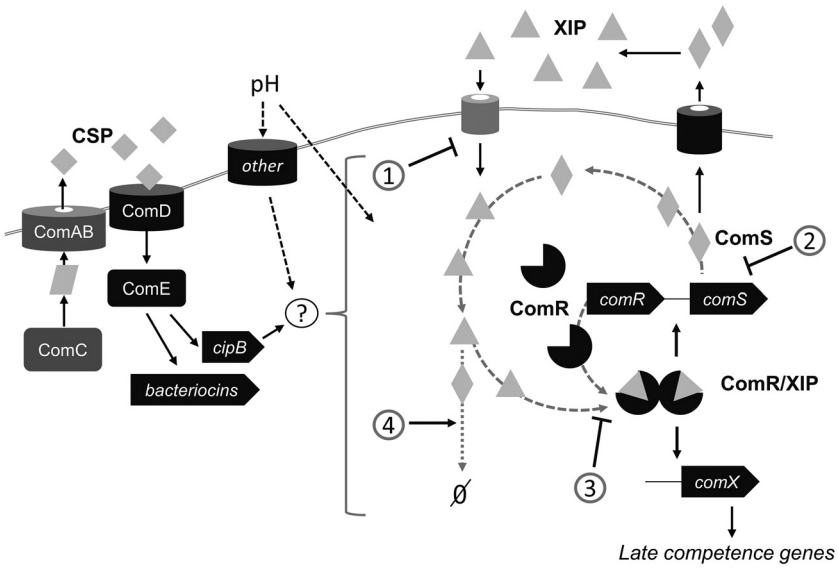

FIG 7 CSP and XIP activation of *comX* (10, 23), and some possible models for the interaction of low pH with the ComR/ComS feedback system that controls P_{comX}. ComS is processed to form XIP, which forms with ComR a complex that is the immediate activator of P_{comX}. We have previously hypothesized that ComR may form an activating complex with either XIP or ComS (10). CSP activates the ComD/ComE two-component system, which in turn stimulates the ComR/ComS system, although the connecting pathway is not known. Low pH could potentially suppress P_{comX} activity by inhibiting import of exogenous XIP (scenario 1), inhibiting expression of *comS* (scenario 2), inhibiting formation of the ComR/ComS or ComR/XIP complex that activates transcription of both *comS* and *comX* (scenario 3), or enhancing degradation of ComS and/or XIP (scenario 4). (Adapted from reference 10 with permission of John Wiley & Sons [copyright 2012 Blackwell Publishing Ltd.].)

sponds unimodally to XIP, with the entire population activating P_{comX} within the pH window. In the model of Son et al. (10), the bimodal (CSP) and unimodal (XIP) responses result from two different modes of activation of the ComR/ComS system: stochastic autofeedback activation by CSP and direct activation by XIP, respectively (Fig. 7). Unimodal activation occurs because exogenous XIP directly stimulates formation of the ComR/XIP complex, which is the immediate transcriptional activator of P_{comX}. It has been proposed that bimodal (CSP-induced) activation relies on fluctuations in the activity of the ComR/ComS autofeedback system, which lead in some cells to a self-activated state that triggers P_{comX} (10, 40, 41).

A second difference between CSP and XIP activation is that, among cells that are activated by CSP, the level of P_{comX} activity (typically 1,000 to 2,000 fluorescence units per activated cell in Fig. 3) is largely insensitive to pH. High or low pH does not suppress P_{comX} activity (in response to CSP) by reducing *gfp* expression in the activated cells; rather, it reduces the number of cells that become activated. Thus, the autofeedback mechanism underlying bimodality in the P_{comX} response evidently still functions at low pH, but the mechanism that drives some cells into the activated state is less effective. In contrast, low pH suppresses the average level of XIP-induced P_{comX} activity in all cells, from about 4,000 to 5,000 fluorescence units per cell at pH 7.1 to about 200 at pH 6.0.

Third, we observed a difference in the ability of high peptide signal concentrations to restore P_{comX} activity. Near pH 6, P_{comX} activity in response to XIP was almost (although not completely) restored to its pH 7 level by a higher concentration of XIP, whereas the response to CSP was not restored at all by CSP concentrations as high as 6 μM. The two peptides interact differently with the cell. XIP must be internalized by an ATP binding cassette transporter, whereas CSP appears to interact with extracellular domains of ComD and does not require uptake. One possible reason for the different responses to higher CSP versus higher XIP could thus have been a loss of sensing or signal transduction of CSP through ComDE. However, the behavior of *cipB* (see Fig. S6 in the supplemental material) shows that the CSP-ComDE circuit, which is the immediate activator of P_{cipB}, remains functional at pH values where P_{comX} is unresponsive to CSP. Further, the response of P_{cipB} to CSP is unimodal, unlike the response of P_{comX}. Therefore, instead of inhibiting the ComDE pathway, low pH may affect the action of XIP and/or ComS within the ComR/ComS feedback loop. Presumably, such an effect would be more readily compensated through direct stimulation by exogenous XIP than through the indirect, upstream action of CSP. Similarly, the kinetic data of Fig. S4 in the supplemental material suggest that the effect of low pH is more proximal to the action of XIP (i.e., to the ComR system) than to the action of CSP (at ComDE).

Figure 7 suggests several possible ways that low pH could restrict the availability of intracellular XIP or its precursor ComS to interact with ComR: low pH may slow the import of exogenous XIP (scenario 1), reduce *comS* expression (scenario 2), inhibit the association of ComR with XIP or ComS (scenario 3), or accelerate the intracellular degradation of XIP or ComS (scenario 4). All of these scenarios would lead to declining P_{comX} activity at low pH.

It is plausible that low pH could restrict the internalization of exogenous XIP, such as by inhibiting expression of the gene for the oligopeptide permease (*opp*), as in scenario 1. The effect of pH on XIP internalization remains to be explored. However, while this scenario could explain the pH dependence of the XIP response, it would not explain our CSP data, as the bimodal CSP response does not require XIP import. An *opp* mutant that lacks any ability to import XIP was still able to activate P_{comX} bimodally in response to CSP (10). Therefore, a pH-dependent change in *opp* expression

is insufficient to explain the observed suppression of the bimodal CSP response at low pH.

Similarly, a pH-sensitive inhibition (Fig. 7, scenario 2) of ComS synthesis could explain the CSP results but not the XIP results. While the declining production of ComS at low pH would make it less likely for the ComR/ComS autofeedback loop to activate under CSP stimulation, P_{comX} activation by XIP does not require ComS synthesis. In particular, we previously showed that exogenous XIP activates P_{comX} via ComR even in *comS*-deficient mutants (10). Inhibited production of ComS at low pH would not explain the loss of responsivity to XIP.

However, scenarios 3 and 4 in Fig. 7 could both potentially explain the XIP and CSP data. Both would lead to a reduced intracellular concentration of the transcriptional activator complex at low pH, where this reduction could be alleviated to some extent by higher concentrations of exogenous XIP. In both scenarios 3 and 4, the activity of the CSP/ComD/ComE pathway at low pH would have a less direct effect on the ComR/ComS system and so additional CSP may be insufficient to reactivate that system.

The possibility that low extracellular pH favors degradation of XIP or inhibits its binding to ComR suggests that ComS overexpression would potentially restore the activity of P_{comX} at low pH. This idea is intriguing in light of the behavior of strains of *S. mutans* that were engineered to overproduce ComS (11, 27). In these strains growing in defined media, genetic competence was observed for a much longer duration than in the wild-type strain, with competence extending into late exponential or early stationary phase.

A very simple pH-sensitive association of ComR with XIP is at least in principle compatible with the observed narrow pH window for XIP response, as demonstrated in Fig. S7 in the supplemental material. The intracellular pH decreases with the extracellular pH, and *S. mutans* employs several mechanisms, such as upregulation of molecular chaperones and DNA repair enzymes, to tolerate low intracellular pH (42, 43). However, *S. mutans* can also actively export protons using the F_1F_o ATPase (44), so the interior of the cell can be maintained at a substantially more alkaline pH than the exterior. At an extracellular pH of 5.0, glycolyzing *S. mutans* was able to maintain a ΔpH of 1.37 ± 0.09 (45). Therefore, it is possible that in our low-pH studies *S. mutans* maintains an intracellular pH that is less acidic than the environment. The observed effects of extracellular pH on competence regulation may then occur via signaling pathways that sense the extracellular pH, rather than through direct pH effects on interactions within the ComR/ComS system. For example, the efficiency of the ComR/ComS system, including perhaps ComS or XIP degradation at extreme pH, could fall under the active control of an environmentally sensitive signaling system such as LiaFRS, CiaRH, BdsRM, HdrRM, or others (8, 46). In addition, *vicR* expression is sensitive to CSP and was shown to be pH dependent, and its regulation was sensitive to LiaFRS, which may act as a pH sensor (47). Several of the scenarios enumerated above could plausibly fall under the control of such a pH-sensing system.

Therefore, multiple and potentially overlapping circuits could contribute to our observations, and a more detailed genetic and biochemical investigation of the interaction between pH and other regulators is required. Our data clearly point toward a pH dependence in the autofeedback strength in the ComR/ComS system, but distinguishing between the many possible mechanisms is a complex task that lies outside the scope of the present study. The task will most likely include (e.g.) investigation of the pH dependence of DNA binding by the ComR/XIP complex or the stability of that complex. Targeted mutagenesis of ComR residues involved in formation of the complex or DNA binding (48), as well as manipulation of pH-sensitive signaling pathways, would also be useful.

We previously showed that very small changes in the composition of the growth media can generate large, qualitative changes in the regulation of genetic competence in *S. mutans*. Here we have found that the activation of this system by either XIP or CSP is also very sharply tuned to extracellular pH, showing again that *S. mutans* exerts meticulous control of virulence behaviors in response to environment. Our data may have interesting consequences for spatial and temporal control of the transformability and virulence of *S. mutans* in oral biofilms, where local pH and chemical and physical conditions are highly heterogeneous and evolve during growth (49). In immature biofilms, peptides such as XIP and CSP may diffuse readily while pH changes rapidly, leading to greater but transient stimulation of competence and associated virulence behaviors as *S. mutans* competes with other biofilm flora. In contrast, in more mature and cariogenic biofilms, the poor diffusibility of the peptides and the sharply reduced local pH would inhibit activation of the competence network in many regions throughout the biofilm, thus limiting entry into the competent state among better-established cells.

ACKNOWLEDGMENTS

We acknowledge funding support from NIDCR under awards 1R01DE023339 and 1R01DE13239.

REFERENCES

1. **Fontaine L, Wahl A, Fléchard M, Mignolet J, Hols P.** 2015. Regulation of competence for natural transformation in streptococci. Infect Genet Evol **33:**343–360. http://dx.doi.org/10.1016/j.meegid.2014.09.010.
2. **Johnston C, Martin B, Fichant G, Polard P, Claverys J.** 2014. Bacterial transformation: distribution, shared mechanisms and divergent control. Nat Rev Microbiol **12:**181–196. http://dx.doi.org/10.1038/nrmicro3199.
3. **Perry D, Kuramitsu HK.** 1981. Genetic-transformation of *Streptococcus mutans*. Infect Immun **32:**1295–1297.
4. **Perry D, Wondrack LM, Kuramitsu HK.** 1983. Genetic transformation of putative cariogenic properties in *Streptococcus mutans*. Infect Immun **41:**722–727.
5. **Cvitkovitch D.** 2001. Genetic competence and transformation in oral streptococci. Crit Rev Oral Biol Med **12:**217–243. http://dx.doi.org/10.1177/10454411010120030201.
6. **Smith EG, Spatafora GA.** 2012. Gene regulation in *S. mutans*. J Dent Res **91:**133–141. http://dx.doi.org/10.1177/0022034511415415.
7. **Li Y, Tian X.** 2012. Quorum sensing and bacterial social interactions in biofilms. Sensors **12:**2519–2538. http://dx.doi.org/10.3390/s120302519.
8. **Ahn S, Wen ZT, Burne RA.** 2006. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect Immun **74:**1631–1642. http://dx.doi.org/10.1128/IAI.74.3.1631-1642.2006.
9. **Ahn S, Wen ZT, Burne RA.** 2007. Effects of oxygen on virulence traits of *Streptococcus mutans*. J Bacteriol **189:**8519–8527. http://dx.doi.org/10.1128/JB.01180-07.
10. **Son M, Ahn S, Guo Q, Burne RA, Hagen SJ.** 2012. Microfluidic study of competence regulation in *Streptococcus mutans*: environmental inputs modulate bimodal and unimodal expression of *comX*. Mol Microbiol **86:**258–272. http://dx.doi.org/10.1111/j.1365-2958.2012.08187.x.
11. **Guo Q, Ahn S, Kaspar J, Zhou X, Burne RA.** 2014. Growth phase and pH influence peptide signaling for competence development in *Streptococcus mutans*. J Bacteriol **196:**227–236. http://dx.doi.org/10.1128/JB.00995-13.
12. **Ahn S, Kaspar J, Kim JN, Seaton K, Burne RA.** 2014. Discovery of novel peptides regulating competence development in *Streptococcus mutans*. J Bacteriol **196:**3735–3745. http://dx.doi.org/10.1128/JB.01942-14.
13. **Kaspar J, Ahn S, Palmer SR, Choi SC, Stanhope MJ, Burne RA.** 2015.

A unique ORF within the *comX* gene of *Streptococcus mutans* regulates genetic competence and oxidative stress tolerance. Mol Microbiol **96:**463–482. http://dx.doi.org/10.1111/mmi.12948.
14. **Stewart PS, Franklin MJ.** 2008. Physiological heterogeneity in biofilms. Nat Rev Microbiol **6:**199–210. http://dx.doi.org/10.1038/nrmicro1838.
15. **Burne RA, Marquis RE.** 2000. Alkali production by oral bacteria and protection against dental caries. FEMS Microbiol Lett **193:**1–6. http://dx.doi.org/10.1111/j.1574-6968.2000.tb09393.x.
16. **Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates JR, III, Heydorn A, Koo H.** 2012. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathog **8:**e1002623. http://dx.doi.org/10.1371/journal.ppat.1002623.
17. **Filoche S, Wong L, Sissons CH.** 2010. Oral biofilms: emerging concepts in microbial ecology. J Dent Res **89:**8–18. http://dx.doi.org/10.1177/0022034509351812.
18. **Koo H, Falsetta ML, Klein MI.** 2013. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. J Dent Res **92:**1065–1073. http://dx.doi.org/10.1177/0022034513504218.
19. **Matsui R, Cvitkovitch D.** 2010. Acid tolerance mechanisms utilized by *Streptococcus mutans*. Future Microbiol **5:**403–417. http://dx.doi.org/10.2217/fmb.09.129.
20. **McNeill K, Hamilton IR.** 2003. Acid tolerance response of biofilm cells of *Streptococcus mutans*. FEMS Microbiol Lett **221:**25–30. http://dx.doi.org/10.1016/S0378-1097(03)00164-2.
21. **Lee MS, Morrison DA.** 1999. Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. J Bacteriol **181:**5004–5016.
22. **Aspiras MB, Ellen RP, Cvitkovitch DG.** 2004. ComX activity of *Streptococcus mutans* growing in biofilms. FEMS Microbiol Lett **238:**167–174. http://dx.doi.org/10.1111/j.1574-6968.2004.tb09752.x.
23. **Mashburn-Warren L, Morrison DA, Federle MJ.** 2010. A novel double-tryptophan peptide pheromone controls competence in *Streptococcus* spp. via an Rgg regulator. Mol Microbiol **78:**589–606. http://dx.doi.org/10.1111/j.1365-2958.2010.07361.x.
24. **Hossain MS, Biswas I.** 2012. An extracellular protease, SepM, generates functional competence-stimulating peptide in *Streptococcus mutans* UA159. J Bacteriol **194:**5886–5896. http://dx.doi.org/10.1128/JB.01381-12.
25. **Lemme A, Grobe L, Reck M, Tomasch J, Wagner-Dobler I.** 2011. Subpopulation-specific transcriptome analysis of competence-stimulating-peptide-induced *Streptococcus mutans*. J Bacteriol **193:**1863–1877. http://dx.doi.org/10.1128/JB.01363-10.
26. **Wenderska IB, Lukenda N, Cordova M, Magarvey N, Cvitkovitch DG, Senadheera DB.** 2012. A novel function for the competence inducing peptide, XIP, as a cell death effector of *Streptococcus mutans*. FEMS Microbiol Lett **336:**104–112. http://dx.doi.org/10.1111/j.1574-6968.2012.02660.x.
27. **Desai K, Mashburn-Warren L, Federle MJ, Morrison DA.** 2012. Development of competence for genetic transformation by *Streptococcus mutans* in a chemically defined medium. J Bacteriol **194:**3774–3780. http://dx.doi.org/10.1128/JB.00337-12.
28. **Malone CL, Boles BR, Lauderdale KJ, Thoendel M, Kavanaugh JS, Horswill AR.** 2009. Fluorescent reporters for *Staphylococcus aureus*. J Microbiol Methods **77:**251–260. http://dx.doi.org/10.1016/j.mimet.2009.02.011.
29. **Lauderdale KJ, Malone CL, Boles BR, Morcuende J, Horswill AR.** 2010. Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. J Orthop Res **28:**55–61. http://dx.doi.org/10.1002/jor.20943.
30. **Terleckyj B, Willett NP, Shockman GD.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect Immun **11:**649–655.
31. **Kwak IH, Son M, Hagen SJ.** 2012. Analysis of gene expression levels in individual bacterial cells without image segmentation. Biochem Biophys Res Commun **421:**425–430. http://dx.doi.org/10.1016/j.bbrc.2012.03.117.
32. **Tuson HH, Weibel DB.** 2013. Bacteria-surface interactions. Soft Matter **9:**4368–4380. http://dx.doi.org/10.1039/c3sm27705d.
33. **Perry JA, Jones MB, Peterson SN, Cvitkovitch DG, Lévesque CM.** 2009. Peptide alarmone signalling triggers an auto-active bacteriocin necessary for genetic competence. Mol Microbiol **72:**905–917. http://dx.doi.org/10.1111/j.1365-2958.2009.06693.x.
34. **Dufour D, Cordova M, Cvitkovitch DG, Lévesque CM.** 2011. Regulation of the competence pathway as a novel role associated with a streptococcal bacteriocin. J Bacteriol **193:**6552–6559. http://dx.doi.org/10.1128/JB.05968-11.
35. **Chen JD, Morrison DA.** 1987. Modulation of competence for genetic transformation in *Streptococcus pneumoniae*. J Gen Microbiol **133:**1959–1967.
36. **Li Y, Lau P, Lee J, Ellen R, Cvitkovitch D.** 2001. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol **183:**897–908. http://dx.doi.org/10.1128/JB.183.3.897-908.2001.
37. **Tian X, Dong G, Liu T, Gomez ZA, Wahl A, Hols P, Li Y.** 2013. MecA protein acts as a negative regulator of genetic competence in *Streptococcus mutans*. J Bacteriol **195:**5196–5206. http://dx.doi.org/10.1128/JB.00821-13.
38. **Merritt J, Zheng L, Shi W, Qi F.** 2007. Genetic characterization of the hdrRM operon: a novel high-cell-density-responsive regulator in *Streptococcus mutans*. Microbiology **153:**2765–2773. http://dx.doi.org/10.1099/mic.0.2007/007468-0.
39. **Merritt J, Qi F.** 2012. The mutacins of *Streptococcus mutans*: regulation and ecology. Mol Oral Microbiol **27:**57–69. http://dx.doi.org/10.1111/j.2041-1014.2011.00634.x.
40. **Smits WK, Kuipers OP, Veening J.** 2006. Phenotypic variation in bacteria: the role of feedback regulation. Nat Rev Microbiol **4:**259–271. http://dx.doi.org/10.1038/nrmicro1381.
41. **Raj A, van Oudenaarden A.** 2008. Nature, nurture, or chance: stochastic gene expression and its consequences. Cell **135:**216–226. http://dx.doi.org/10.1016/j.cell.2008.09.050.
42. **Lemos JA, Burne RA.** 2008. A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiology **154:**3247–3255. http://dx.doi.org/10.1099/mic.0.2008/023770-0.
43. **Cotter PD, Hill C.** 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol Mol Biol Rev **67:**429–453. http://dx.doi.org/10.1128/MMBR.67.3.429-453.2003.
44. **Bender GR, Sutton SV, Marquis RE.** 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. Infect Immun **53:**331–338.
45. **Dashper SG, Reynolds EC.** 1992. pH regulation by *Streptococcus mutans*. J Dent Res **71:**1159–1165. http://dx.doi.org/10.1177/00220345920710050601.
46. **Tremblay YDN, Lo H, Li Y, Halperin SA, Lee SF.** 2009. Expression of the *Streptococcus mutans* essential two-component regulatory system VicRK is pH and growth-phase dependent and controlled by the LiaFSR three-component regulatory system. Microbiology **155:**2856–2865. http://dx.doi.org/10.1099/mic.0.028456-0.
47. **Li Y, Lau P, Tang N, Svensater G, Ellen R, Cvitkovitch D.** 2002. Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. J Bacteriol **184:**6333–6342. http://dx.doi.org/10.1128/JB.184.22.6333-6342.2002.
48. **Parashar V, Aggarwal C, Federle MJ, Neiditch MB.** 2015. Rgg protein structure-function and inhibition by cyclic peptide compounds. Proc Natl Acad Sci U S A **112:**5177–5182. http://dx.doi.org/10.1073/pnas.1500357112.
49. **Ahn S, Ahn S, Browngardt CM, Burne RA.** 2009. Changes in biochemical and phenotypic properties of *Streptococcus mutans* during growth with aeration. Appl Environ Microbiol **75:**2517–2527. http://dx.doi.org/10.1128/AEM.02367-08.